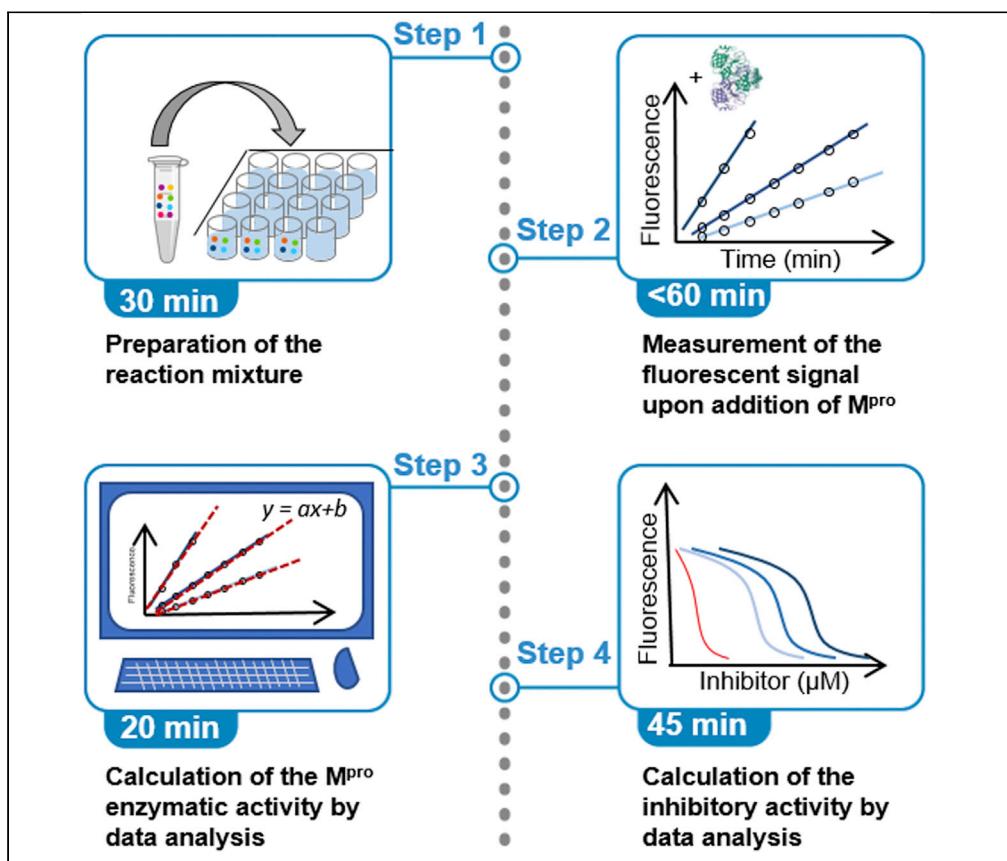


Protocol

Fluorogenic *in vitro* activity assay for the main protease M^{pro} from SARS-CoV-2 and its adaptation to the identification of inhibitors



This protocol describes an *in vitro* fluorogenic assay to measure the proteolytic activity and identify inhibitors of M^{pro}, the main protease produced by SARS-CoV-2. Studies to identify potential inhibitors of M^{pro} mainly rely on *in silico* molecular dynamics simulations or on FRET substrates. The protocol is based on an aminomethyl coumarin substrate. High sensitivity, specificity, and an easily detectable fluorescent read-out are the advantages offered by this rapid assay, which allows high throughput screening of new M^{pro} inhibitors.

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Highlights

M^{pro} is the main protease produced by SARS-CoV-2

An *in vitro* fluorogenic assay for the measurement of the proteolytic activity of M^{pro}

An *in vitro* fluorogenic assay for the inhibitor screening of M^{pro}

High sensitivity, specificity, and an easily detectable fluorescent read-out

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Protocol

Fluorogenic *in vitro* activity assay for the main protease M^{Pro} from SARS-CoV-2 and its adaptation to the identification of inhibitorsJulien Ihssen,^{1,2,*} Greta Faccio,^{1,3,*} Chunyan Yao,¹ Teja Sirec,¹ and Urs Spitz¹¹Biosynth Carbosynth, Rietlistrasse 4, 9422 Staad, Switzerland²Technical contact³Lead contact*Correspondence: julian.ihssen@biosynth-carbosynth.com (J.I.), greta.faccio@biosynth-carbosynth.com (G.F.)
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SUMMARY

This protocol describes an *in vitro* fluorogenic assay to measure the proteolytic activity and identify inhibitors of M^{Pro}, the main protease produced by SARS-CoV-2 (Severe acute respiratory syndrome coronavirus 2). Studies to identify potential inhibitors of M^{Pro} mainly rely on *in silico* molecular dynamics simulations or on FRET (Fluorescence Resonance Energy Transfer) substrates. The protocol is based on an aminomethyl coumarin substrate. High sensitivity, specificity, and an easily detectable fluorescent read-out are the advantages offered by this rapid assay, which allows high throughput screening of new M^{Pro} inhibitors.

BEFORE YOU BEGIN

This protocol details an *in vitro* fluorogenic assay to measure the activity of the main protease produced by SARS-CoV-2 (Severe acute respiratory syndrome coronavirus 2) and its application to the evaluation of M^{Pro} inhibitory compounds. The protocol assays the M^{Pro} protease activity using a specific fluorogenic probe Ac-Abu-Tle-Leu-Gln-AMC which has been described by [Rut et al. \(2020\)](#) as the preferred synthetic substrate for M^{Pro} ($K_M = 207 \mu\text{M}$ for analog substrate Ac-Abu-Tle-Leu-Gln-ACC). The fluorogenic substrate molecule contains the two non-natural amino acids L-2-aminobutyric acid (Abu) and L-tert-leucine (Tle) and the two natural amino acids L-leucine (Leu) and L-glutamine (Gln). The oligopeptide carries the fluorophore 7-Amino-4-methylcoumarin (AMC) at the C-terminal end of glutamine, i.e., linked via a peptide bond ([Figure 1](#)). Upon cleavage by M^{Pro}, a fluorogenic moiety is released and a fluorescent signal is produced. The assay can be used for, e.g., the analysis of M^{Pro} activity in infected cell cultures and assessment of potential M^{Pro} inhibitors.

Prior to the experiment, the assay buffer and the stock solutions of substrate, enzyme, and inhibitors should be prepared to ensure a straightforward assembly of the reaction mix in the multiwell plate before measurement.

Before you start, familiarize yourself with the material safety data sheets (MSDS) for all the reagents.

Note: Fluorescence can be expressed in arbitrary units (AU), relative fluorescence units (RFU) or sometimes simply abbreviated as FL and its values are device-dependent ([Beal et al., 2018](#)).

Prepare fluorogenic substrate solution

⌚ Timing: 10 min



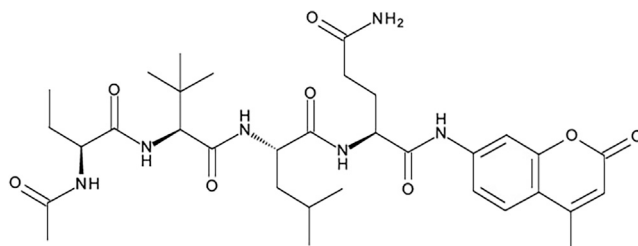


Figure 1. Chemical structure of the synthetic fluorogenic substrate Ac-Abu-Tle-Leu-Gln-AMC for the main protease M^{Pro} of SARS-CoV-2.

A stock solution (5 mM or 3.28 mg/mL) of the fluorogenic substrate Ac-Abu-Tle-Leu-Gln-AMC (M.W. 656.77 g/mol) should be prepared and can be stored as described for future use.

1. Weigh 1–2 mg of Ac-Abu-Tle-Leu-Gln-AMC in a microcentrifuge tube using an analytical balance.
2. Add an exact volume of dimethyl sulfoxide (DMSO, calculated from weighing) to reach a final concentration of 3.28 mg/mL Ac-Abu-Tle-Leu-Gln-AMC, e.g., add 0.5 mL of DMSO to 1.74 mg of substrate.
 - a. Vortex briefly to dissolve.
3. Store stock solution at -20°C , protected from light. The solution is stable under these conditions for at least one year.

Preparation of the M^{Pro} stock solution

⌚ Timing: 10 min

Stock solutions of SARS-CoV-2 main protease (final concentration 1 mg/mL or 28 μM) can be prepared as follows:

4. Reconstitute the M^{Pro} lyophilisate directly in the vial of the supplier, i.e., containing 1 mg, by adding 1 mL of 50% v/v glycerol in water.
 - a. Dissolve any solid completely by gently pipetting up and down avoiding the formation of bubbles ([Troubleshooting 2](#)).
5. Divide in 0.1 mL aliquots of stock solution.
6. Store at a temperature between -15°C and -25°C and avoid freeze/thaw cycles, i.e., prepare aliquots for a single use. The solution is stable under these conditions for at least one year.

Preparation of the assay buffer

⌚ Timing: 30 min

The assay buffer contains 20 mM (2.42 g/l) TRIS, 1 mM EDTA, 150 mM NaCl, 1 mM DTT and has a pH of 7.3. It can be prepared as follows for a 100 mL final volume:

7. Weigh 242 mg tris(hydroxymethyl)aminomethane (TRIS, M.W. 121.14 g/mol) and place it in a beaker containing approximately 80 mL of ultrapure water and a magnetic stirrer.
 - a. Dissolve by stirring.
8. Weigh 37.2 mg of ethylenediaminetetraacetic acid tetrasodium salt tetrahydrate (EDTA, tetrasodium salt, M.W. 452.23 g/mol).
 - a. Add it to the TRIS-containing solution and stir to dissolve.
9. Weigh 876 mg of NaCl.
 - a. Add it to the TRIS-containing solution and stir to dissolve.
 - b. Adjust pH to 7.3 by adding drop-wise 1 N HCl.

10. Add ultrapure water to reach a volume of 100 mL in a measuring cylinder.
 - a. Stir with a magnetic bar for 1 min.
11. In a separate tube, weigh an amount between 200 and 400 mg of dithiothreitol (DTT, M.W. 154.253 g/mol).
 - a. Dissolve it in an exact volume of ultrapure water (calculated from weighing) to achieve a 1 M stock solution (154.25 mg/mL).
12. To prepare the buffer for activity measurement, add 1 μ L DTT stock solution per mL buffer, e.g., add 5 μ L of DTT stock solution to 5 mL of assay buffer.

Reagent	Final concentration	Amount
TRIS	20 mM	242 mg
EDTA	1 mM	37.2 mg
NaCl	150 mM	876 mg
DTT	1 mM	100 μ L (1 μ L of 1M DTT stock solution per mL buffer)
Ultrapure water	-	Up to 100 mL

△ CRITICAL: For best results, use freshly prepared buffer in your experiments. DTT has a half-life of less than one hour under certain conditions in aqueous solution, i.e. 40°C and pH 8.5, and should always be added freshly to solutions. DTT stock solution can be stored in aliquots for up to one year at -80°C , but multiple freezing and thawing cycles should be avoided. The addition of EDTA to the DTT stock solution at a 0.1 mM concentration improves its stability (Stevens et al. 1983).

Alternatives: Buffers alternative to TRIS that have been used with MP^{Pro} include 20 mM HEPES, pH 6.0, 0.4 mM EDTA, 1 mM DTT, 1% glycerol that has been used when assaying the activity with a FRET (fluorescence resonance energy transfer) substrate (Hung et al. 2020).

△ CRITICAL: DTT is often used in inhibition assays with proteases, but may interfere with the efficacy of certain inhibitors, e.g. ebselen, disulfiram, tideglusib, carmofur, shikonin, and PX-12 (Ma et al. 2020).example, ebselen. In the initial screenings of potential inhibitors and in control assays with ebselen, it is recommended to omit DTT from the assay buffer or test different reducing agents (Lee et al. 2012). DTT-free assay buffer can be stored at $+4^{\circ}\text{C}$ for several months. Similarly, EDTA prevents the possible interference of metal ions with the protease and thus compromises its activity (Kozak et al. 2020).

Preparation of the stock solutions of the inhibitor ebselen

⌚ Timing: 10 min

Ebselen (M.W. 274.2 g/mol) is used here as a positive inhibition control and solutions of additional potential inhibitors to be tested should be prepared similarly and ahead of time. For the preparation of the ebselen stock solution:

13. Weigh an amount of ebselen between 5 and 7 mg in a 2 mL microcentrifuge tube.
14. From the weight, calculate the exact volume of DMSO required for a final 20 mM concentration (5.48 mg/mL).
 - a. Add the calculated volume of DMSO.
 - b. Dissolve by vortexing (Troubleshooting 4).
15. Stock solution can be stored for one year at -20°C .

△ CRITICAL: Concentration of inhibitor stock solution should be at least 200-fold higher than highest final concentration to be tested in the in assay to avoid any potential interference of the solvent.

Preparation of the dilution series of inhibitor

⌚ Timing: 10 min

By supplementing the assay buffer with an inhibitory molecule, the assay measurement can be used to assess the impact of the compound tested on the M^{Pro} protease activity. A high concentration of ebselen can be used as a positive control for activity inhibition. Here, suggested concentrations are presented. M^{Pro} inhibition can be presented as a relative value to the signal obtained without ebselen that will be considered as 0% inhibition (100% activity) reference value. The IC₅₀ value is the inhibitor concentration where the enzyme activity is inhibited by 50%. An IC₅₀ of 0.67 μM for ebselen has been experimentally determined towards SARS-CoV-2 M^{Pro} (Jin et al. 2020). The inhibitory activity of ebselen is tested in a 0.02–20 μM concentration range, with solutions dispensed in triplicate wells. For each novel inhibitor the optimal concentration range, covering the IC₅₀, needs to be determined individually. In order to account for assay variability, we recommend to prepare and measure three replicate dilution series for each inhibitor.

The serial dilution of the inhibitors should be prepared right before measurement, possibly while the instrument is equilibrating to the desired temperature of 37°C.

The stock solution of inhibitor, i.e., ebselen, prepared in advance, can be diluted in a time-efficient way directly in the multiwell plate as follows:

16. In three 1.5 mL microcentrifuge tubes, combine 0.998 mL of assay buffer without DTT (in the case of ebselen) and 2 μL of 200× inhibitor stock solution (20 mM ebselen). This 2× ebselen working solution has a concentration of 40 μM.
 - a. Mix well by vortexing.
17. Prepare wells with decreasing ebselen concentrations as follows (Table 1):
 - a. Add 0.2 mL of each 2× ebselen working solution (40 μM) to wells A1, B1, C1 of a black multiwell plate with black bottom.
 - b. Add 0.1 mL assay buffer without DTT to Wells A2-A12, B2-B12, and C2-C12 using a multi-channel pipette.
 - c. Transfer 0.1 mL of solution from wells A1, B1, C1 to wells A2, B2, C2.
 - d. Mix with new pipette tips by pipetting up and down 5 times, then transfer 0.1 mL to Wells A3, B3, and C3.
 - e. Mix with new pipette tips, then transfer 0.1 mL to adjacent wells to the right.
 - f. Continue this procedure until wells A11, B11, C11 are reached.
 - g. Remove 0.1 mL from wells A11, B11, C11 after mixing.
18. Prepare control wells without M^{Pro} (background fluorescence).
 - a. Add 0.1 mL 2× ebselen working solution (40 μM) to well D1.
 - b. Add 0.1 mL assay buffer without DTT to well D2.

⚠ **CRITICAL:** use new pipette tips for each step to avoid cross contamination.

Preparation of the stock solution of 7-amino-4-methylcoumarin (AMC)

⌚ Timing: 10 min

Table 1. Plate layout for the serial dilutions of ebselen; final concentration in assay is indicated (μM)

Row/Column	1	2	3	4	5	6	7	8	9	10	11	12
A	20	10	5	2.5	1.25	0.625	0.313	0.156	0.078	0.039	0.02	0
B	20	10	5	2.5	1.25	0.625	0.313	0.156	0.078	0.039	0.02	0
C	20	10	5	2.5	1.25	0.625	0.313	0.156	0.078	0.039	0.02	0
D	20	0										

AMC is the fluorogenic moiety released by the M^{Pro} proteolytic activity and it is here used to obtain a conversion factor between the fluorescence values measured by the fluorimeter and the concentration of substrate consumed by the enzymatic reaction.

To prepare a 10 mM stock solution of 7-Amino-4-methylcoumarin (AMC, M.W. 175.18 g/mol):

19. Weigh between 10 and 20 mg, i.e., approximately 17.5 mg, of AMC in a 10 mL measuring flask, note the exact weighed amount.
20. Add assay buffer to a final volume of 10 mL.
 - a. Vortex to dissolve ([Troubleshooting 2](#)).
 - b. Calculate the exact concentration of stock solution from the weight
21. Use freshly prepared during the day and store on ice when not in use.

Experimental setup

⌚ Timing: 15 min

Use a plate reader with fluorescence function, e.g., SpectraMax M5 plate reader (black multiwell plate, PMT setting “low”, 0.2 mL assay volume). In general, ensure that the reading settings are optimal by measuring a solution of AMC with the same concentration as the maximum final concentration achievable during the assay; measurement conditions under which this solution does not saturate the detector of the plate reader are suitable. Assays can be done in plate readers with or without temperature control. If a plate reader with temperature control is used, assays are best performed at 37°C, mimicking conditions of viral replication in human hosts.

Once the plate reader has reached temperature set point, place the plate in the reader and pre-incubate for 10 min in order to equilibrate to the desired assay temperature.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Ac-Abu-Tle-Leu-Gln-AMC	Biosynth Carbosynth	FA178674
AMC	Biosynth Carbosynth	FA00826
DTT	Biosynth Carbosynth	FD02370
EDTA, tetrasodium salt	Biosynth Carbosynth	FE04031
Ebselen	Biosynth Carbosynth	FE75162
Glycerol	Merck Millipore	137028
HCl	Sigma-Aldrich	H9892
NaCl	Sigma-Aldrich	S7653
SARS-CoV-2 main protease	Biosynth Carbosynth	BS178678
TRIS	Biosynth Carbosynth	FT15751
Other		
Black 96-well plate	Thermo Fisher, Nunc	137101
SpectraMax M5 plate reader	Molecular Devices	0310-5625

MATERIALS AND EQUIPMENT

Alternatives: In our study, we used a SpectraMax M5 plate reader (Molecular Devices) but other models are also suitable for the assay to be performed. Software able to fit the

data obtained and produce slope values indicative of the protease activity, i.e. variation of fluorescence per min, such as Microsoft Excel are useful and time-savers.

Alternatives: Measurements should be performed in black multiwell plates with black bottom to avoid crosstalk among wells. The use of black plates is recommended as it helps to reduce the background signal noise; opaque white plates are an alternative but conditions might need to be tuned as the background signal will be more evident.

STEP-BY-STEP METHOD DETAILS

Assembly of the assay mixture for M^{Pro} activity measurement

⌚ Timing: 10 min

Depending on the extent of your experiment and the number of samples to measure, plan the number of wells of the multiwell plate you will need and the volume of reagents required, i.e., considering a single reaction volume of 200 μ L but also a possible 10% disposable volume. To measure M^{Pro} activity in three replicate measurements, we have routinely used a working solution containing the substrate at a concentration of 100 μ M. This working solution is prepared as follows:

1. Prepare the 1.025 \times substrate working solution by adding 20.5 μ L of substrate stock solution to 979.5 μ L of assay buffer with DTT.
2. After adding the assay buffer and the substrate but before the addition of the enzyme, pre-warm the plate for 5 min at the desired assay temperature in the plate reader, e.g., 22°C or 37°C.
3. Before measurement, let all solutions equilibrate to room temperature (20°C–22°C).
4. Dispense 195 μ L of the 1.025 \times working solution in each of 5 wells of a black multiwell plate.

After adding the assay buffer and the substrate but before the addition of the enzyme, pre-warm the plate for 5 min at the desired assay temperature in the plate reader, e.g., 22°C or 37°C.

Measurement of the enzymatic activity of the M^{Pro} protease

⌚ Timing: 10–60 min

Enzymatic activity is defined as enzyme units and one enzyme unit corresponds to the amount of enzyme that will release one micromole of 7-amino-4-methylcoumarin (AMC) in one minute in assays with 0.1 mM Ac-Abu-Tle-Leu-Gln-AMC at 37°C and pH 7.3. In this case, the enzymatic activity can be expressed as increase of fluorescence (arbitrary units, RFU) over time. Most microplate readers allow the analysis of the curves produced by the measurements and a slope value expressed in variation of fluorescent signal per minute can be obtained. These values can be used to compare results obtained within the same measurement or by the same instrument, i.e., in RFU/min.

For routine activity assays and inhibition studies, the comparison of fluorescence values as RFU is sufficient. In this respect, a positive control reaction is set up and can be used as a reference of 100% relative to the other reactions.

In the procedure, the enzyme mixture is added last to initiate the reaction, the measurement should start immediately (Figure 2) with settings enabling fluorescence measurement from the top with an excitation wavelength of 380 nm and an emission wavelength of 455 nm every 30 s for at least 10 min.

Upon preparation of the working solution, the fluorogenic reaction is started by the addition of the protease.

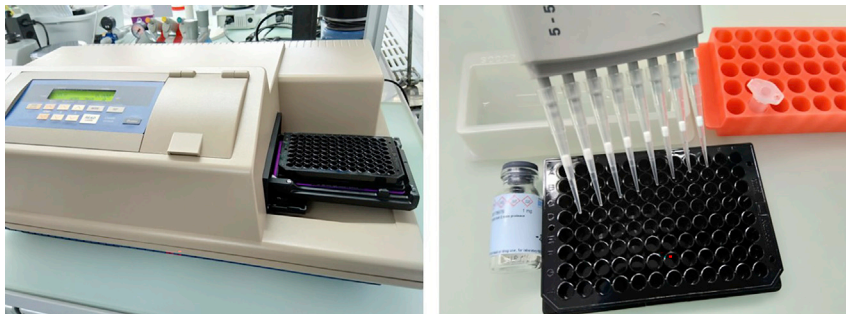


Figure 2. Required Equipment

Image of the SpectraMax M5 plate reader (left) and photos taken during the activity measurement (right), i.e., multi-channel pipette, black multiwell plate, vial with protease stock solution, polypropylene tube for preparation of working solution and assay buffer (in the reservoir). The use of pipette tips with filter is facultative.

5. To each well containing the substrate working solution as previously described:
 - a. Add 5 μL of sample containing active 25 $\mu\text{g}/\text{mL}$ M^{Pro} for a final concentration of 0.025 $\mu\text{g}/\text{mL}$.
 - b. Mix by pipetting up and down several times avoiding bubble formation.
6. Proceed to measure the fluorescence immediately.
 - a. Place the microplate in the plate reader and measure the fluorescence from the top with an excitation wavelength of 380 nm and an emission wavelength of 455 nm every 30 s over a 10–60 min period until sufficient data points for a reliable data fitting are gathered.
 - b. Shaking during measurement is an option some equipment offer and is beneficial to ensure a more homogenous distribution of both substrate and enzyme.
7. Export the data obtained as fluorescence intensity (RFU or AU) vs. time ([Troubleshooting 1](#)).

Calculation of the enzymatic activity by data analysis

⌚ Timing: 20 min

After collecting the data on the development of fluorescence ([Figure 3](#)) upon addition of M^{Pro} to the substrate working solution, analysis of the curves allows for the calculation of the enzymatic activity in relative or absolute values. The spectrophotometer proprietary software might allow a first-degree polynomial fitting (straight line) of the curves obtained and the direct export of the slope values. Otherwise, this can be performed in Microsoft Excel as follows:

8. Plot fluorescence intensity values versus time using a scatter plot.
 - a. Set the time scale to minutes.
9. Select at least 5 adjacent points covering more than 1 min in the initial part of the curve for the fitting and plot them separately.
10. Add a linear trendline (straight line) to the data and choose the option to display curve equation and R-squared value on chart.
 - a. Include as many adjacent points as possible and aim for an R-squared above 0.98 ([Figure 3](#), [Figure 4](#)).
11. Record the slope value as the activity value in RFU/min ([Troubleshooting 3](#)).

Measurement of M^{Pro} inhibition

⌚ Timing: 10–60 min

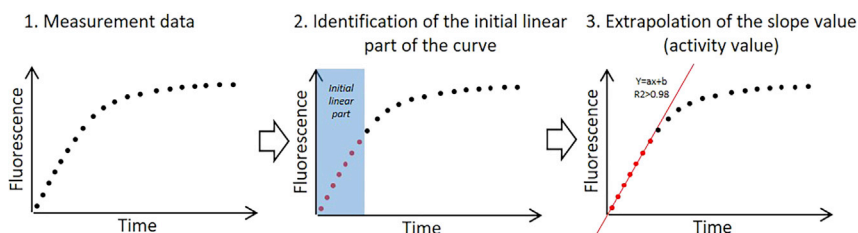


Figure 3. Example of scatter plot, identification of the linear part of the curve to obtain the slope value corresponding to the enzymatic activity value as variation of fluorescence over time

In order to save reagent while maintaining a substrate concentration below the reported K_M , the inhibitor screening assays are performed at 25 μM fluorogenic substrate concentration and 5 $\mu\text{g/mL}$ M^{Pro} concentration by diluting the stock solution 200 \times , i.e., 100 μL of respective stock solutions to 1.9 mL of buffer. The assay is performed in a black multiwell plate and in a preheated plate reader at 37°C to simulate human body conditions. When scouting for novel inhibitors, the step here presented with ebselen should be adapted to include a wider inhibitor concentration range between 1 nM and 1 mM and through \log_{10} dilution steps.

12. Add 80 μL of working solution M^{Pro} to the previously described setup of diluted inhibitor in wells A1-A12, B1-B12 and C1-C12 using a multichannel pipette.
13. Add 80 μL assay buffer to negative control wells D1 and D2 for protease-free control reactions.
14. Pre-warm the plate for 5 min at 37°C by placing it in the plate reader.
15. Add 20 μL of the 10 \times fluorogenic substrate to all wells using a multichannel pipette and mix by pipetting up-and down 3 times. Use new pipette tips for every well.
16. Measure fluorescence immediately from the top with excitation wavelength at 380 nm and emission wavelength of 455 nm for at least 10 min in a kinetic mode.
 - a. Highest concentration of inhibitor ebselen in wells A1, B1, C1 is 20 μM , concentration decreases serially by factor two from wells A/B/C2 to A/B/C11.
 - b. Wells A12, B12 and C12 correspond to uninhibited M^{Pro} activity.

Data analysis of M^{Pro} inhibition study

⌚ Timing: 45 min

The spectrophotometer proprietary software might allow a first degree polynomial fitting (straight line) of the curves obtained and the direct export of the slope values (Figure 5). These values are activity values, e.g., 265.78 RFU/min in Figure 5. Otherwise, the data can be analyzed in Microsoft Excel as previously presented (section above) to obtain the variation of fluorescence per minute (enzymatic activity value).

After collecting the data on the development of fluorescence upon addition of M^{Pro} in the presence of both the substrate and the inhibitor, the calculation of the inhibitory activity is carried out in software such as Microsoft Excel.

17. By plotting the fluorescence values (RFU) over time and fitting the curve to a straight line, extract the rate of RFU increase over time (RFU/min) for each well using the trend line fitting in Microsoft Excel (Figure 3).
 - a. Show the trend line equation and R^2 fitting value.
 - b. In all uninhibited and partially inhibited wells, RFU increase should be linear over the whole measurement period. The correlation coefficient for these wells should be above 0.98.
18. Control wells (D1, D2) without M^{Pro} should show low RFU values which do not increase over time.

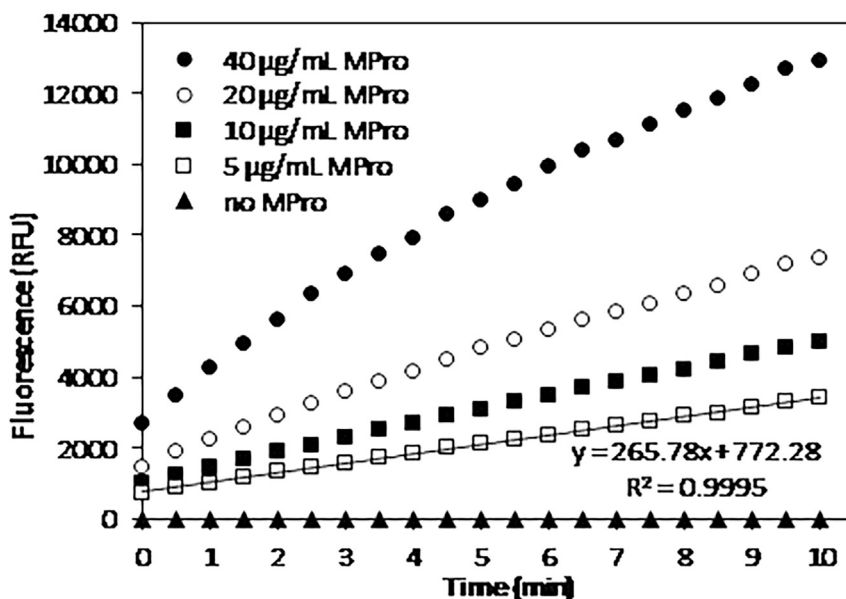


Figure 4. Development of the fluorescent signal over time upon incubation of 0.1 mM fluorogenic substrate Ac-Abu-Tle-Leu-Gln-AMC with MPro at different concentrations. The protease-free control sample is reported as filled triangles.

- a. Wells at inhibitor concentrations higher than a 10-fold IC₅₀ concentration or higher should show no or only marginal RFU increase.
 - b. Calculate the average activity values and corresponding standard deviations of all wells with similar inhibitor concentration using the respective functions in Microsoft Excel, e.g., average values of results from wells A1-B1-C1.
19. Divide the average activity, i.e., slope of the curve in RFU/min, of wells with inhibitor by the calculated average slope of samples without inhibitor.
- a. Calculate the relative activity as percentage (%) by multiplying by 100. The relative inhibition value can be obtained by subtracting this activity from 100, i.e., a calculated

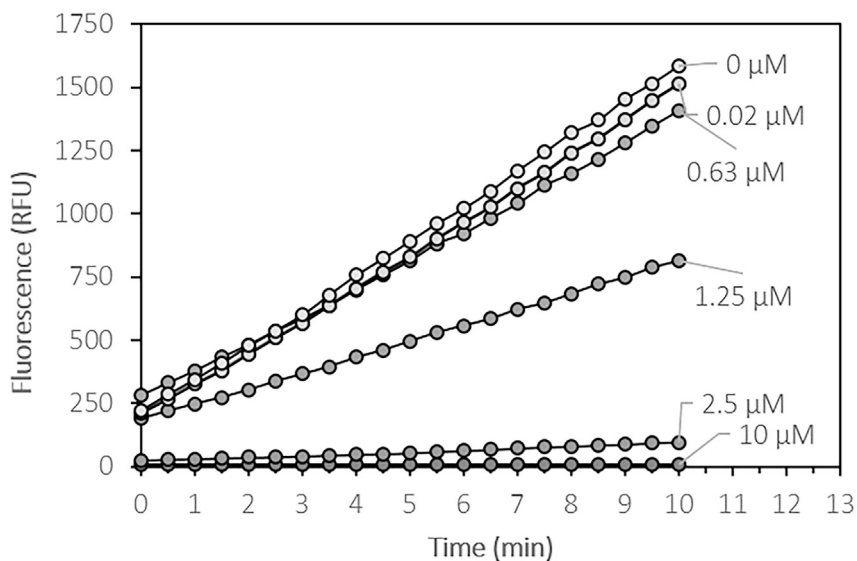


Figure 5. Fluorescence development curves over time in the presence of different esbelen concentrations.

Table 2. Example of dilutions used for the preparation of the calibration curve

AMC final concentration (μM)	Dilution factor	Volume of 100 μM AMC solution per well (μl)	Volume of assay solution per well (μl)	Total volume (μl)
25	4	50.0	150.0	200
12.5	8	25.0	175.0	200
6.25	16	12.5	187.5	200
3.125	32	6.2	193.7	200
1.5625	64	3.1	196.9	200
0	-	0	200.0	200

10% relative activity in the presence of the inhibitor corresponds to a 90% relative inhibition.

- b. Calculate the relative standard deviation by dividing the standard deviation of the slopes of replicate wells by the corresponding standard deviation of the average of the corresponding uninhibited wells and multiplying by 100.
20. Draw inhibition curve using a scatter graph in Microsoft Excel, i.e., the x-axis presents the inhibitor concentration in a μM concentration and the y-axis presents the relative inhibition as percentage (%).
 - a. Rescale to a logarithmic scale on the x-axis.
21. Calculate a rough estimation of the IC_{50} value by interpolation between data points above and below the 50%; for more precise values fit with a sigmoidal curve ([Troubleshooting 5](#)).

Note: For the determination of IC_{50} with the highest accuracy, it is recommended to use multiple dilution steps with smaller changes ($\text{IC}_{50} \pm 10\text{--}30\%$) in a second experiment, i.e. to achieve a higher resolution.

Absolute quantification of the amount of substrate transformed

⌚ Timing: 60 min

When the enzymatic activity needs to be expressed in enzyme units, i.e., U/mL or U/mg, a conversion needs to be carried out. The conversion factor is specific for each fluorimeter and can be obtained by preparing a calibration curve of AMC, the fluorophore produced by the enzymatic reaction ([Table 2](#), [Figure 6](#)).

22. The stock solution of ± 10 mM AMC is first diluted in assay buffer to reach a 100 μM concentration.
 - a. Calculate exact volume (± 100 μL) of AMC stock solution to reach a final concentration of 100 μM in 10 mL total volume
 - b. Add calculated volume of AMC stock solution to a 10 mL measuring flask and fill to exactly 10 mL with assay buffer.
 - c. Store in the dark and use fresh.
23. In a black microplate, prepare a series of solutions with varying 7-Amino-4-methylcoumarin (AMC, M.W. 5.18 g/mol) concentration ranging from 1.5 μM to 25 μM , see [Table 2](#) as example. Include a negative control well with assay buffer only. Each measurement should be performed at least in triplicates, e.g., three replicate wells per concentration.
24. Measure the fluorescence intensity of the solutions containing different amounts of AMC with the same settings used for the activity assay.
 - a. Export the values in fluorescence units, e.g., RFU or AU.
25. Plot them against the corresponding concentration of AMC.
 - a. Fit the data points in the linear part of the curve and obtain the equation of the fitting curve ([Figure 6](#)).

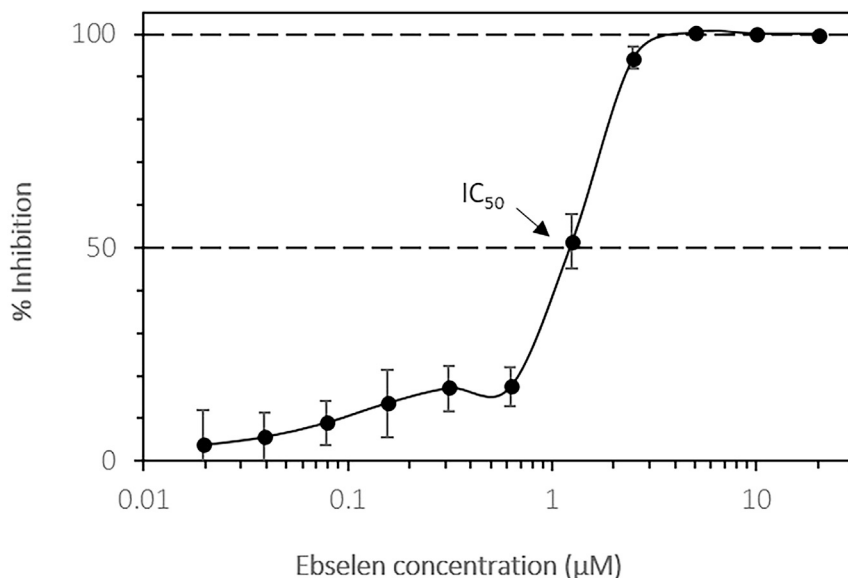


Figure 6. Inhibition curve of ebselen and M^{Pro} protease obtained by plotting the enzymatic activity in fluorescence variation over time vs. inhibitor concentration. Measurements were carried out in triplicate and the average is reposted with the corresponding standard deviation (error bars).

26. This equation, i.e., the slope coefficient 555.86 RFU/μM in Figure 7, can be used to convert the instrument-specific fluorescence values to the concentration of AMC in the assay, resulting from conversion of substrate.

△ **CRITICAL:** Each measurement should be performed at least in duplicate, e.g. two identical wells per condition and an average value calculated and used for the curve fitting. Aim for an R² value above 0.98.

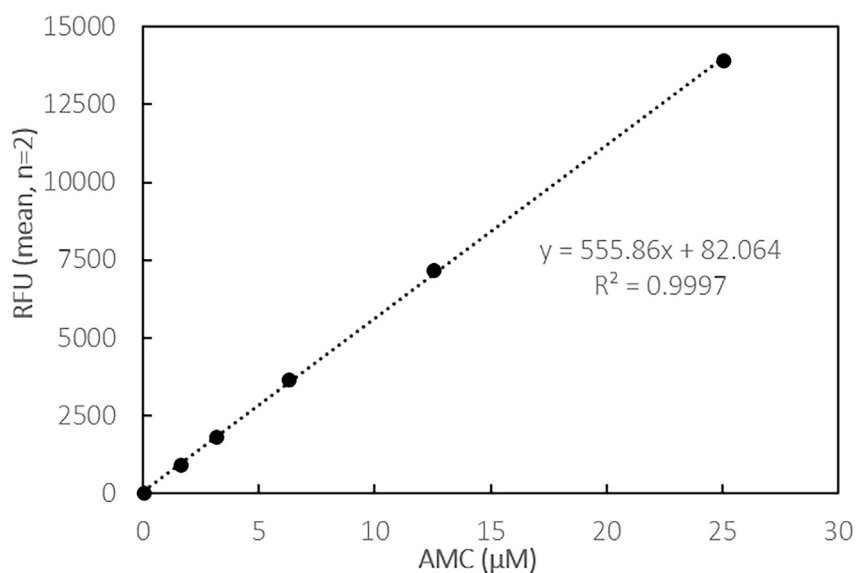


Figure 7. Example of standard curve of the fluorescent moiety AMC alone in assay buffer and data measured with the SpectraMax M5 plate reader (black multiwell plate, PMT setting “low”, 0.2 mL assay volume)

This result is device-dependent.

Table 3. Example of raw data obtained from the testing of the inhibitory action of ebselen

Well (ebselen, μM)	A1 (20)	A2 (10)	A3 (5)	A4 (2.5)	A5 (1.25)	A6 (0.63)	A7 (0.31)	A8 (0.16)	A9 (0.08)	A10 (0.04)	A11 (0.02)	A12 (0, control)
Time (min)	Fluorescence (RFU)											
0.0	11	10	13	25	189	282	299	308	197	211	211	221
0.5	11	9	13	28	219	331	350	371	246	267	266	285
1.0	11	10	13	30	248	379	398	419	301	319	325	344
1.5	11	10	13	34	273	432	453	481	355	372	379	408
2.0	11	10	12	36	303	482	505	531	414	432	444	477
2.5	10	10	13	38	337	536	561	582	474	491	507	537
3.0	11	10	12	41	365	584	614	641	528	551	567	600
3.5	11	10	12	45	395	639	670	705	587	609	635	679
4.0	11	10	12	48	431	700	734	763	647	678	704	756
4.5	11	10	12	50	461	758	790	819	724	751	768	822
5.0	11	10	11	54	496	814	846	871	770	808	827	891
5.5	11	9	11	59	530	881	906	937	833	865	900	961
6.0	12	10	11	63	558	922	961	989	886	930	964	1021
6.5	11	9	11	66	585	982	1024	1042	945	1001	1025	1090
7.0	12	10	11	72	622	1040	1086	1113	1016	1089	1100	1171
7.5	12	10	10	77	647	1111	1153	1183	1089	1156	1162	1242
8.0	11	10	11	80	683	1159	1209	1247	1140	1224	1239	1319
8.5	11	10	11	85	720	1216	1262	1293	1198	1303	1297	1370
9.0	11	9	10	87	748	1280	1329	1350	1257	1356	1371	1453
9.5	11	9	10	93	786	1346	1398	1428	1323	1432	1446	1515
10.0	11	10	11	96	814	1408	1479	1484	1387	1503	1512	1585
Calculated slope (RFU/min)	0.02	-0.02	-0.28	7.23	63	112	116	117	120	130	130	137
R2	0.201	-0.24	-0.90	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99	0.99

EXPECTED OUTCOMES

Successfully carried out, this protocol allows the detection and measurement of the proteolytic activity of the previously referred to as main protease M^{Pro} of SARS-CoV-2 as a fluorescent signal (Figure 3). By supplementing potential inhibitors to the activity assay, their inhibitory activity on M^{Pro} protease can be assessed. The number of natural molecules acting as inhibitors is growing (Bharadwaj et al. 2021; Ghahremanpouret al. 2020, Qiao et al. 2021) and the assay can serve as a tool for the high throughput screening of new inhibitors to the clinically relevant protease M^{Pro} (Figure 4) and the determination of their characteristic IC_{50} values (Table 3, Figure 6). Moreover, it presents how it is possible to convert device-specific fluorescence values into values reflecting the substrate concentration (Figure 3, Table 4).

LIMITATIONS

This protocol relies on the high specificity of the fluorogenic substrate used for M^{Pro} . It has however been tested only with the purified form of the M^{Pro} protease and thus results obtained from complex M^{Pro} -containing samples might differ. In studies analyzing activity of M^{Pro} in infected cells, an antibody-based assay specific for M^{Pro} might be used to further support the evidence obtained with the fluorescence-based assay presented here.

TROUBLESHOOTING

Problem 1

No fluorescent signal is detected upon addition of the enzyme to the substrate solution during the measurement of the enzymatic activity (step 6).

Table 4. Example of fluorescence data obtained for the preparation of the standard curve correlating AMC concentrations and fluorescence values

AMC concentration (μM)	Replicate	Fluorescence (RFU)	Mean fluorescence (RFU)
25	A	13917	13903
	B	13889	
12.5	A	7070	7172
	B	7273	
6.25	A	3620	3648
	B	3675	
3.125	A	1798	1825
	B	1852	
1.5625	A	9123	911
	B	909	
0	A	11	8.8
	B	5	

Potential solution

Ensure to be using a fresh M^{Pro} solution, dispense a higher concentration of M^{Pro} in the assay mixture, increase the detection sensitivity of the reader, or monitor the reaction for longer time. Additionally, you might want to test different concentrations of protease and of substrates at first to assess the sensitivity of the detection of your instrument, e.g., being able to detect the signal of a solution containing an AMC concentration as expected to be produced by the reaction without saturating the detector. For example, to obtain concentration of M^{Pro} main protease lower than 1 mg/mL, pre-dilute M^{Pro} solution in assay buffer, e.g., 0.1 mL of stock solution added of 0.9 mL of assay buffer for a 10-fold dilution.

Problem 2

The substrate, inhibitor, or enzyme are not soluble during the preparation of the stock solutions (steps 1–15 in “before you begin”).

Potential solution

Control the pH of the buffer for protease dilution and check if a suitable organic solvent such as DMSO has been used for the substrate

Problem 3

No linearity is found in the development of fluorescence during the measurement of the enzymatic activity (step 10).

Potential solution

Repeat the measurement by adjusting the reading settings of the instrument or by using higher or lower concentration of M^{Pro} protease, e.g., 10-fold higher or lower, or measure for a longer time, e.g., 1 h.

Problem 4

The inhibitor is not soluble in DMSO and precipitates giving a cloudy solution during the preparation of the stock solutions (step 14 in “before you begin”).

Potential solution

Depending on the chemical properties of investigated inhibitors use other solvents, e.g., water, ethanol, methanol, or dimethyl formamide, and mild heating may be better suited.

Problem 5

No S-shaped curve is visible after plotting the activity data obtained in the presence of an inhibitor during the inhibition study (step 20).

Potential solution

Ensure that the dilution series encompasses the IC₅₀ value, if known, for the inhibitor used as reference, e.g., ebsele to be used as positive control. As previously mentioned referring to unknown inhibitors, the IC₅₀ might lay outside the presented range. In this case, an initial step to test inhibition and encompassing a concentration between 1 nM and 1 mM of the inhibitor through log₁₀ dilution steps should be performed.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Greta Faccio, greta.faccio@biosynth-carbosynth.com.

Materials availability

This study did not generate new unique reagents, strains or plasmids. All described materials are commercially available without use restrictions.

Data and code availability

This study did not generate code and datasets.

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AUTHOR CONTRIBUTIONS

I.J. curated the conceptualization, data analysis, investigation, writing, review, and editing; Y.C. contributed to assay development; F.G. and S.T. curated the writing, original draft, writing, review, and editing; S.U. curated the conceptualization, resources, writing, review, and editing.

DECLARATION OF INTERESTS

I.J., F.G., Y.C., and S.U. are employed by Biosynth AG, a company part of the Biosynth Carbosynth group, a corporation that markets the enzyme and substrate used in this protocol. This does not alter our adherence to the journal's policies on sharing data and materials. No further conflicts of interest are declared.

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